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## IMPROVING THE TECHNIC OF THE FEULGEN STAIN

J. A. DE TOMASI, *Commission on Standardization of Biological Stains, Geneva, N. Y.*

ABSTRACT.—**A study has been made of the Feulgen stain, in which the staining fluid is a decolorized basic fuchsin. Particular attention has been given to the variation in behavior of different fuchsin samples, the reagent to be employed in decolorizing the dye, the acidity of solutions, and the value of several counterstains. A modified procedure is suggested, the details of which are given in the paper. The principle modifications of earlier procedures which are recommended are as follows: the use of a specially purified pararosanilin as a dye; the employment of $K_2S_2O_5$ instead of $NaHSO_3$ as a decolorizing agent; and counterstaining with fast green in the case of plant tissue or with orange G for animal material.**

### INTRODUCTION

The Feulgen procedure, which is now coming into prominence as a cytological stain, was originally developed by Feulgen and Rossenbeck (1924) as a microchemical test. It was designed to distinguish that type of nucleic acid found in chromatin (which contains a hexose radical) from other similar acids which contain pentose instead of hexose groups. The Feulgen reaction is based upon the use of a reduced (colorless) form of basic fuchsin, which develops a specific purple color in contact with an aldehyde.

As the ending "al" is used in the Geneva chemical terminology to indicate a compound of aldehydic nature, it is quite natural that Feulgen should have called his stain a "Nuclealreaktion." It is generally assumed, as many have stated, that it is quite a sharp reaction based upon the liberation of free aldehyde groups by hydrolysis of the thymonucleic acid from the chromatin. While its sharpness and significance may still be open to discussion, no doubt the high degree of specificity and the clean yet delicate differentiation it affords are of great appeal to the biomicroscopist. In spite of such advantages, however, it has never been as widely used as many other

nuclear stains that do not possess its inherent good qualities. Accordingly, an investigation has been carried on by the Stain Commission in hopes of simplifying and standardizing the procedure so as to bring it into more general use. The present paper deals with the staining technic; a paper by Scanlan and Melin, to appear in the next issue, will discuss the question of the proper dye to employ.

## The Method and its Modifications

A survey of the literature shows a great many variations from the original method as given by Feulgen and Rossenbeck (1924). The original directions included, for instance, a reagent (1% dimethylcyclohexandione, a cycloparaffin derivative) which has since been dropped by the majority of those who worked with the stain. The purpose of adding this to the descending alcohols was to bind the aldehydes always present in alcohol and prevent the oxidizing effect of atmospheric oxygen on them. These compounds were supposed to be harmful and detrimental to the action of the stain. The original procedure, among other things, included also a combination of steps for the hydrolization of the thymonucleic acid with warm HCl. Furthermore, the preparation of the stain solution and the rinsing bath to follow it called for sodium bisulfite (i.e., acid sodium sulfite, $NaHSO_3$) as a source of $SO_2$ for the reduction of the dye. Altho the general scheme of the original method has been followed by later workers, nevertheless, a great number of variations have been introduced which at times do not seem to warrant the innovation. Such changes or new interpretations involve timing, concentration of the reagents, and various other factors. This becomes evident by consulting any of the outstanding papers on the Feulgen stain of the last few years. See, for instance, the work of Westbrook (1930), Boas and Biechele (1932), Milowidow (1933), and Margolena (1932). The last-named author, for example, gives the following schedule:

*Preparation of fuchsin solution:* Dissolve 1 g. basic fuchsin by bringing to boiling in 200 cc. dist. water. Cool to 50° C., filter, add 20 cc. 0.5 *N* HCl. Cool to 25° C., add 1 g. sodium bisulfite.

*Staining:* Run down to water; 0.5 *N* HCl, 2 min. or more; 0.5 *N* HCl at 60° C., 2 min. or more; rinse in cold HCl; dip in water. Stain in fuchsin, 1–3 hr.; wash in 3 baths of the following: 200 cc. dist. water, 10 cc. 0.5 *N* HCl, 10 cc. 10% sodium bisulfite. Rinse in dist. water, run up thru the alcohols, counterstain with any of the usual counterstains, dehydrate, clear and mount.

In the light of these directions, a brief discussion follows of the major features of the method, as preliminary to an explanation of those changes now proposed as experimentally sound and distinctly advisable.

## Experimental

*Preparation of Fuchsin Solution:* The purpose of dissolving the dye in a solution of the kind in question is to produce a stain temporarily "bleached" or colorless which will work selectively and be fixed specifically by the nuclear matter. It is obvious that the theory is very much the same as in the case of Schiff's reagent: a solution of magenta (fuchsin) is barely decolorized with $SO_2$; if an aldehyde is present in any solution to which this reagent is added, a bright pink color soon appears. Ketones do not give the same reaction.

Table 1. Data Concerning Decolorized Solutions of Various Fuchsin Samples

| Sample No. | pH | Age | Color of Solution | | Staining |
|---|---|---|---|---|---|
| | | | Fresh | Aged as in Column 3 | |
| | | | EXPERIMENTAL SAMPLES | | |
| 1 | 1.63 | 8 months | Light straw | Yellow | Excellent |
| 2 | 1.69 | 8 months | Yellow brown | Red brown | Quite good |
| 3 | 1.70 | 8 months | Pink brown | Deep pink | Not good |
| 4 | 1.75 | 8 months | Pink brown | Deep red pink | Too dark |
| 5 | 1.52 | 8 months | Purplish pink | Deep red pink | Not good |
| 6 | 1.62 | 8 months | Straw yellow | Deep red pink | Excellent |
| 7 | 1.75 | 8 months | Light straw | Red pink | Satisfactory |
| 8 | 1.35 | 4 months | Light straw | Red pink | Satisfactory |
| 9 | 1.40 | 4 months | Light straw | Red pink | Satisfactory |
| 10 | 1.33 | 4 months | Light straw | Red pink | Satisfactory |
| | | | COMMERCIAL SAMPLES | | |
| 11 | 1.33 | 6 months | Gold yellow | Red pink | Mediocre |
| 12 | 1.58 | 4 months | Deep orange | Red pink | Satisfactory |
| 13 | 1.42 | 4 months | Light straw | Red pink | Excellent |
| 14 | 1.45 | 4 months | Yellow straw | Red pink | Satisfactory |

There have been tested in this laboratory nearly 80 samples of basic fuchsin, some commercial samples submitted by stain companies, others experimental products from the Industrial Farm Products Research Division at Washington. The object was to learn which one of these samples would decolorize satisfactorily to a light yellow color, would give the least precipitate and a correct tinting of the chromatin in the Feulgen technic. It was found that only a few of the commercial samples were altogether satisfactory and that products from the same source would vary greatly from time to time. Commercial basic fuchsins vary greatly in composition (Conn, et al., 1936, pp. 99 to 103), so that it is difficult to be certain what any given sample may be. On the other hand, after much work

at Washington, the laboratory there has furnished a set of *pararosanilin* samples which proved to be exceedingly good.

With this heterogeneous collection of samples it proved interesting to learn what the H-ion concentration was and whether there were any variations detectable which could be correlated with other significant data. It was expected to find the hydrogen-ion concentration quite high, but it was a matter of some surprise to discover how little variation there is in the reaction of the stain solutions. Table 1 is representative of the whole group. Determinations are by potentiometer, with a gold and quinhydrone electrode.

It is evident that when these solutions are fresh the H-ion concentration is quite uniform, in spite of the marked differences displayed in appearance and behavior. It is of interest also to note that after a period of time, due undoubtedly to inadequate sealing of bottles, there is a darkening of the solutions with a tendency to turn red or pink. That is accompanied by a small but marked rise of the pH, linked with a loss in the $SO_2$, followed eventually by reabsorption of atmospheric oxygen.

The question of the optimum acidity in the solution is still debatable and calls for further investigation, which the writer hopes to undertake. So far the experience in this laboratory has been that the use of HCl in the dye solution is necessary with our American fuchsins. Lack of sufficient acidity causes foggy or smudged stains affecting the whole section, and the chromatin does not seem to differentiate and hold the stain as firmly as desirable.

Another point which should be discussed here is the matter of the sulfite. It was realized nearly two years ago that the reducing agent used was not actually $NaHSO_3$ (acid sodium sulfite) but rather the metabisulfite $Na_2S_2O_5$. The acid sulfite is extremely difficult to keep anhydrous and it is only available when specially prepared. The metabisulfite is the most closely related salt available commercially, altho still a product subject to a certain degree of decomposition. Potassium metabisulfite ($K_2S_2O_5$) is still more stable and uniform in composition, and was thought to be closely enough related to the sodium salts to warrant investigation. It has now been in use for more than a year in comparison with the homologous sodium salt in amounts which are comparable, 0.6 g. of $K_2S_2O_5$ to to 0.5 g. of $Na_2S_2O_5$ for 100 cc. of the dye solution. The substitution of a different cation in the salt does not seem to impair the performance of the stain in any detectable way and gives us a much better tool for a closer control of the reaction. At the present time 0.5 g. of $K_2S_2O_5$ is used to 100 cc. of the dye solution, an amount which has

proved sufficient for a complete decolorization in 12 hours. It may be added that recent correspondence from England tells of some investigations carried out there on very much the same lines and with comparable results.[1]

In the preparation of the solution it will be noticed that, as given in Margolena's schedule, the fuchsin is to be brought to a boil. This and other schemes have been tried and it is the writer's conviction that, in order to get as much as possible of the dye in solution, a better technic is to bring the water to the boiling point and to pour it onto the dye in a large-mouthed receptacle, to shake it up for a little while, to cool, and to proceed with the filtration. The second step of cooling to 25° C. is of no significance for quantities of approximately 100 cc.

*Staining:* As to a pretreatment we find that with the fuchsins available now there is no need for a special treatment of the section with dimethylcyclohexandione. The dyes are sufficiently selective and the reducing agent is powerful enough to eliminate whatever traces of the color might have clung to the cytoplasm.

While it has been suggested by others that 0.5 *N* HCl be used thruout, there is evidence that a *normal* solution as originally prescribed is more reliable. Some of the fuchsins in the present series do not respond as readily and neatly when the acidity in which the hydrolization and washing are carried out is unduly lowered.

The matter of the timing of the various steps deserves special consideration. An attempt has been made to establish quite definite limits for each one of them. It was found that plant materials generally require more time than animal tissues. This is particularly true with respect to the hydrolization process and the staining proper. A complete schedule as used in our laboratory, with all working details, is given below. The use of carefully prepared distilled water thruout is especially important. When a good Feulgen stained section comes out of the staining solution, it must show a definite faint purplish tinge. This is maintained thru the three sulfite washings and the color on the chromatin may finally be enhanced by a short soaking (10–20 minutes) in plain distilled water. Sections carried so far can be kept overnight in the water without noticeable damage.

*Counterstaining:* It has been suggested in the past that most of the common counterstains are satisfactory in the case of a Feulgen stain. Erythrosin, light green, and orange G have all been suggested. There are three main vehicles for a counterstain: water, alcohol, or

[1]An account of this work is given in Revector Bull. of Stain Technique (published by Vector Manufacturing Co., London), **2**, No. 3, 1936.

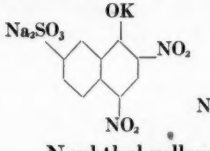
oil (usually clove oil). In this work the matter has been studied with the idea of meeting two major requirements: first, to obtain a counterstain which would permit maximum contrast; second, to secure a dye which would be readily soluble in water and as little as possible in alcohol.

To meet the first point, only green and yellow dyes were considered, as these colors are complementary to purple and magenta. Of the green dyes, light green was soon replaced with fast green, which is only half as soluble in alcohol and is much more resistant to fading. It can be used in 1% aqueous solution or 0.5% in 95% alcohol. In the alcoholic solution it is quite satisfactory for plant tissues, applied directly for two minutes after the last water step. In this case it must be remembered that water would remove it instantly; for this reason the extra dye must be washed out while completing the dehydration with 95% and absolute alcohol. Of the yellow dyes, orange G in 1% aqueous solution for five minutes proves very satisfactory on animal tissues, while it is not taken well by plant

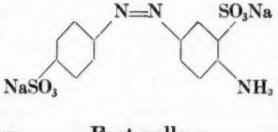
TABLE 2. COUNTERSTAINS INVESTIGATED

| Name | Dye Group | C.I. No. | % Solubility | | Staining |
|---|---|---|---|---|---|
| | | | Water | Alc. | |
| 1. Martius yellow | Nitro | 9 | 4.57 | 0.16 | Tends to replace nuclear stain. |
| 2. Naphthol yellow | Nitro | 10 | 8.96 | 0.025 | Nuclear stain noticeably covered. |
| 3. Fast yellow | Azo | 16 | 18.40 | 0.24 | Too dark, smudgy. |
| 4. Alizarol yellow | Azo | 36 | 25.84 | 0.04 | Tends to replace nuclear stain. |
| 5. Orange II | Azo | 151 | 11.37 | 0.15 | Too diffused. |

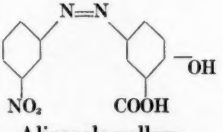
material. As in the case of fast green, water will promptly remove the counterstain; therefore, it must be washed and dehydrated with 95% and absolute alcohol. Less satisfactory results were obtained with 1% aqueous solutions of five other dyes as indicated in Table 2. Of these five dyes the structural formulae of martius yellow and orange II are given in Biological Stains (Conn, et al., 1936). Those of the other three are as follows:




Naphthol yellow




Fast yellow




Alizarole yellow

All these are acid dyes. Alizarole yellow was obtained in the form of its color acid (as shown above) which is not soluble in water, and had to be converted into its Na salt in order to employ it. These

dyes seem to deserve attention and it is hoped to have an opportunity in the near future of devoting more time to them.

It is mentioned above that dyes of high solubility in water should be selected. It is clear that under such conditions one can apply the counterstain after the last water rinse without further complication and with no fear of damage by the subsequent dehydration. This plan has advantages over the much used clove oil dye solutions. In this latter case one must dehydrate completely before applying the counterstain. The counterstaining must then be followed by a wash in xylene which, as in the case of the orange G, causes all the dye to precipitate immediately. This kind of trouble is particularly undesirable in cytological work.

A preliminary note concerning these recommendations has already appeared (de Tomasi, 1936). The detailed steps of the procedure follow.

## Procedure Recommended

*1. Preparation of Fuchsin Solution:*

Dissolve 0.5 g. fuchsin by pouring over it 100 ml. boiling distilled water. Shake thoroly. Cool to 50° C. Filter; add 10 ml. *N* HCl to filtrate. Add 0.5 g. $K_2S_2O_5$. Shake, close tight, store in the dark for 12–18 hours.

*2. Staining:*

Run down to water. Rinse in cold *N* HCl. Hydrolize in *N* HCl at 60° C., 4–5 min. Rinse in cold *N* HCl. Rinse in dist. water. Stain in the decolorized fuchsin (animal tissue 2 hr.; plant tissue 3–5 hr.). Drain and pass quickly to the first of 3 closed Coplin jars containing following acid solution: *N* HCl, 5 ml.; 10% $K_2S_2O_5$, 5 ml.; dist. water, 100 ml. Wash 10 min. in each. Rinse in dist. water. Counterstain: animal tissue 3–5 min. with 1% aq. orange G; plant tissue 1 min. with 0.5% fast green in 95% alcohol. Wipe off excess dye. Wash clean with 95% and dehydrate with absolute alcohol. Clear and mount in dammar.

It is hoped that this revised procedure for the Feulgen stain, together with the new fuchsin to be used in it, will prove a sufficient advance toward perfection of the technic to be of some definite value in histological and cytological work.

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## MICROSCOPIC SLIDES OF CAT TESTIS

THOMAS H. MORTON, *Department of Biology, Niagara University, Niagara Falls, N. Y.*

Thin (3 mm.) slices of cat testis were fixed for 43 hours in freshly prepared Karpechenko's fluid. This fixative was designed for use in plant cytology and was prepared by mixing 50 ml. of 1% aqueous solution of chromic acid, 4.5 ml. of distilled water, 5.0 ml. of glacial acetic acid, and 40 ml. of formalin[1].

Following fixation the material was washed for 24 hours in running water. Dehydration in ethyl alcohol, clearing in toluene, and infiltration with paraffin were carried out gradually. Paraffin (M.P.

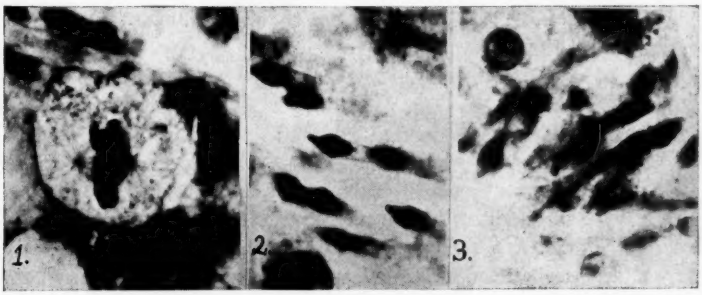


Fig. 1. Metaphase of spermatogonial mitosis. 1250×.

Fig. 2. Spermatid showing deeply stained acrosome, caudal sheath, and derivatives of the centrioles. The derivative of the proximal centriole extends to one side of the caudal sheath. 1250×.

Fig. 3. Later stage of spermiogenesis showing caudal sheath somewhat separated from the spermatid nucleus and in contact with the derivative of the proximal centriole at both sides. 1250×.

51° C.) sections were cut at 5μ and stretched in water at 40° C. on slides coated with Mayer's albumin fixative. After the sections were freed from paraffin they were protected by a thin film of parlodion thruout the remainder of the process. The material was stained in Heidenhain's iron hematoxylin, being mordanted in iron alum for three hours and stained in 0.5% hematoxylin for an equal length of time. Destaining was effected by a 1% aqueous solution of iron alum. The procedure outlined in this paragraph is given in the reference noted below and differs only in the preparation of the staining solution.

[1]Galigher, A. E. 1934. Essentials of Practical Microtechnique in Animal Biology. A. E. Galigher, Inc.

The usual procedure for staining sections in iron hematoxylin was changed only in regard to details of pH control. The usual solution of hematoxylin[2] was prepared. This was saturated with quinhydrone for direct determination of its pH in a quinhydrone electrode. The pH of this solution was then brought within the range from 7.00 to 7.06 by addition of saturated aqueous solution of lithium carbonate and of acetic acid. The regulation of the pH was effected immediately before using the solution. Excellent results were obtained by this method as early as one week after preparation of the hematoxylin solution—3 days after complete solution of the stain.

Fine preparations of mitotic figures were obtained (Fig. 1). The transformation stages of spermiogenesis were exceptionally clear (Figs. 2 and 3). The acrosome retained the stain while the material was destained sufficiently to bring out the finer details in the region of the neck (Fig. 2).

Samples of this material were fixed in Allen's "B-15", 10% formalin in physiological salt, and in Flemming's strong solution with acetic acid. These were treated exactly as and simultaneously with the Karpechenko fixed material, except that longer periods were allowed for mordanting and staining the Flemming fixed tissues. The highest degree of contrast in those stages of spermiogenesis which follow the attachment of the acrosome to the spermatid nucleus was shown in material fixed in Karpechenko's fluid.

The photomicrographs are of sections of cat testis fixed in Karpechenko's fluid and stained as described above. The hematoxylin solution had not acquired the color of port wine before regulation of its pH.

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[2]Hematoxylin (C. P.) Coleman and Bell, Certification No. FH-11.

# MOTOR END-PLATES

J. D. Combs, *Department of Histology and Embryology, University of Georgia, School of Medicine, Augusta, Georgia.*

Abstract.—**In the preparation of motor end-plates for class room work the use of sodium thiosulfate is suggested as a precaution against overstaining and as a regressive measure. Digestion of the connective tissue eliminates the tedious teasing and the possible destruction of the delicate nerve fibers. Mounting in a solution of gum arabic makes the slides more durable than glycerin jelly for class use. Whole neuromuscular spindles may be obtained by this method.**

Preparation of motor end-plates requires considerable time and skill. Experience has proven that the usual gold-chloride-glycerin-jelly mounts for class room use are too fragile to last more than a short time and as a consequence the preparation of these slides must be made a part of the yearly routine. In an effort to eliminate the reduplication of technical procedure, a satisfactory method of preparation of motor end-plates has been used by the author in this laboratory which does not require the use of glycerin jelly as the mounting medium.

Intercostal muscles of a rabbit are obtained immediately after killing. Pieces should be cut small to insure rapid diffusion of the solutions. The staining method is a modification of the Ranvier technic in which small pieces of muscle are placed in fresh lemon juice for 30 minutes. They are rinsed quickly in distilled water, and placed in a bath of 1% gold chloride for 30 minutes. The muscle is washed in distilled water rapidly, and transferred to 10% formic acid for reducing the gold. The reducing must be done in the dark, and will require about six to ten hours. The exact timing must be partly determined by test dissections in glycerin and observation under the microscope. When the endings are well stained, and the muscle is red (not purple), the tissue is washed well in running water, and placed in a 5% solution of sodium thiosulfate for 15 or 20 minutes. Prolonged fixing in this solution tends to destain and, as a consequence, it must be done with care. It may be used as a regressive stage if the tissue is slightly over-stained. The tissue is washed in running water for an hour, and then placed in artificial gastric juice (powdered pepsin, 1 part; water, 1,000 parts; HCl, 3 parts). Digestion takes place better at about body temperature, and the tissue

will be soft in one or two hours. Judgment must be exercised from the texture of the muscle mass. It should be easily spread out with slight pressure under a cover glass. The gastric juice is then washed out by several changes of water. Running water should not be used, since the tissue is extremely flaccid and much of it may be washed away. It is then transferred to pure glycerin, which should be renewed three times in a period of about two hours. Very small pieces of the muscle are cut with scissors and placed on a clean absorbent cloth to drain. Each piece is transferred to a slide and covered with one or two drops of a solution of powdered acacia in water (about the consistency of glycerin) to which a few drops of formalin have been added. By gently pressing a cover glass down on the tissue it will flatten and bring the nerve fibers into view. Observation under the microscope will determine which of the slides have motor end-plates. After a period of time the cover glasses become well fixed, and the slides will stand ordinary handling of balsam and cedar oil mounts.

The slides may be sealed with gold size or thin balsam after they are thoroly dry. This prevents any damage which might be caused by the slides becoming wet. Altho some authors suggest the use of alcohol as a dehydrant, which makes it possible to mount the muscle tissue in balsam, this method was not found to be successful by the author. The lemon juice tends to swell the muscle cells and make them transparent. All available dehydrants caused a shrinkage, which resulted in the cells becoming opaque.

It is advisable to use sodium thiosulfate to prevent the tissue from becoming too dark. The reaction is probably: $2\,AuCl_3 + 8\,Na_2S_2O_3 \longrightarrow Au_2S_2O_3 \cdot 3Na_2S_2O_3 + 6\,NaCl + 2\,Na_2S_4O_6$, in which the gold complex is soluble. The formic acid must be well washed out, however, or there will be a white precipitate of sulfur formed within the tissue. The artificial gastric juice has already been mentioned as a decided advantage over the teasing method and makes it possible to turn out many more slides in a limited time. In the course of developing this technic, entire neuromuscular spindles were isolated. If done on a large scale, it would be possible to obtain them in large numbers.

# A STABLE, HIGH-CONTRAST MORDANT FOR HEMATOXYLIN STAINING

A. G. LANG, *Department of Botany, University of North Carolina, Raleigh, North Carolina*

ABSTRACT.—**Small quantities of sulfuric acid will stabilize iron mordants, used in hematoxylin staining, by preserving these solutions against oxidation. The presence of acetic acid in the mordant improves the specificity of the stain. A stable, high-contrast mordant is obtained when both acids are combined with ferric-ammonium sulfate. This mordant, used in combination with fresh alkaline solutions of hematoxylin, has been found especially effective in the staining of certain nuclear and cytoplasmic components of plant cells.**

In the staining of chondriosomes, plastid primordia, chromonemata, and other structural elements of plant protoplasm with iron alum hematoxylin, an effort is usually made to obtain a maximum high contrast of the stainable structures. While the hematoxylin is generally used in combination with a 4% solution of ferric-ammonium sulfate, a 5% ferric chloride mordant may give equally good contrast. The following modification of the usual mordant has proved to be especially effective for discriminative hematoxylin staining:

| | |
|---|---|
| 4% ferric-ammonium sulfate (C.P.) | 500 cc. |
| Acetic acid (C. P.) | 5 cc. |
| Sulfuric acid (C. P., Sp. Gr. 1.84) | 0.6 cc. |

Altho good results may be obtained by leaving slides in this solution for 2 hours, a 12-hour treatment is recommended.

Destaining is effected with a similar solution, diluted one-half for use:

| | |
|---|---|
| 2% ferric-ammonium sulfate | 500 cc. |
| Acetic acid | 5 cc. |
| Sulfuric acid | 0.3 cc. |

The acetic acid is added to mordant the stainable structures more vigorously and to obtain a greater specificity of the stain. Both cytoplasmic and nuclear components will be stained more precisely and in greater contrast with the more faintly stained background of hyaloplasm and karyolymph. The hematoxylin precipitates heavily in the chromatic material and the finished preparation shows a maximum contrast of black structure against a background which is white, gray, or tan according to the fixative used.

The sulfuric acid is added to prevent the oxidation of ferric sulfate. In theory only a slight excess of $SO_4$-ions will insure a clear solution against precipitates, but in practice the quantity indicated above seems desirable. Such solutions have remained free of precipitates for a year on the shelf and should remain clear indefinitely. No injurious effects have been noted with this type of mordant even in the most delicate structures, in fact equally good results have been obtained when the concentration of this acid is increased to 1%.

Only distilled water is used in preparing the mordants and they are kept in clean, well-stoppered bottles. Aqua regia will quickly remove the iron oxide from containers in which the usual type of mordant has been stored.

The length of time in the stain is modified by too many variables to make a definite schedule possible. In general, the following conditions seem to apply:

With very fresh solutions of 0.5% hematoxylin, the staining time is about 4 to 8 hours. The freshest stains give the highest contrast, the stain becoming less specific with increasing age. Artificially "aged" hematoxylin solutions appear to be undesirable where the very maximum contrast is wanted. In all instances of hematoxylin staining it seems best to leave the slides in the stain for only the shortest effective time. As the hematoxylin solution becomes older, the staining time becomes correspondingly reduced. Noticeably better results are obtained with alkaline hematoxylin[1] than with more acid solutions.

When less contrast and purple tones are desired, the acetic acid is omitted from both mordants. In this case, however, following certain fixatives such as formalin-formic acid and F. A. A., black staining is obtained.

Acetic-mordants are not recommended for general histological work. For such purposes and for general low-contrast staining, where violet and silver-blue tones are especially effective, it seems desirable to use a 4% mordant (without acetic acid) for 2 hours, to stain as usual, and to differentiate with picric acid in 70% ethyl alcohol[2]. Unused stain solutions which are 1 to 3 years old are often quite good for low-contrast staining with a silver-blue effect.

Hutner[3] has recommended the use of $H_2O_2$ as a destaining agent for hematoxylin, this method having been found valuable in work on

[1]Galigher, A. E. 1934. The Essentials of Practical Microtechnic in Animal Biology. A. E. Galigher, Inc., Berkeley, Calif.

[2]Tuan, H. C. 1930. Picric acid as a destaining agent for iron alum hematoxylin. Stain Techn., 5, 135–8.

[3]Hutner, S. H. 1934. Destaining agents for iron alum hematoxylin. Stain Techn., 9, 57–9.

protozoa. One can obtain a very low contrast of the hematoxylin on plant materials by this procedure or by using $FeCl_2$. The $H_2O_2$ can be greatly activated as an oxidant by making it alkaline, but since the peroxide is very unstable in alkaline solution it must be handled cautiously (3% solutions as purchased have a pH of about 2.90 and 30% solutions may have an acidity greater than pH 2.30). The author has not found either acid or alkaline $H_2O_2$ destaining of noteworthy value with plant material, but $FeCl_2$ has been valuable in destaining nucleoli, especially following certain chondriosomal fixatives.

With Delafield's hematoxylin, 70% alcoholic picric acid seems to be decidedly preferable to the usual aqueous picric acid or HCl solutions for differentiation purposes.

# PINACYANOL AS A SUPRA-VITAL MITOCHONDRIAL STAIN FOR BLOOD

DUNCAN C. HETHERINGTON, *Department of Anatomy, Duke University School of Medicine, Durham, N. Carolina.*

ABSTRACT.—**Pinacyanol[1] is an excellent, almost permanent and very selective stain for mitochondria in supra-vital blood preparations. It may be used alone or in combination with neutral red for the study of fresh blood, bone marrow, spleen or lymph node smears. One drop of a 0.1% solution of pinacyanol in absolute alcohol, per cubic centimeter of the same solvent, is a satisfactory dilution for making stain films for blood with an average concentration of cells.**

In 1933 Proescher[2] described briefly the nature, formula and staining qualities of pinacyanol when used as a histological stain for frozen sections of native or fixed tissues. No mention was made of its use as a supra-vital stain.

The writer, while studying some leukaemic blood by the supra-vital method, used pinacyanol among other dyes for the preparation of stain films and found that the mitochondria of the blood cells were selectively stained very deep purple or dark blue. At the same time it was noticed that these structures retained the dye for very long periods of time and that there was no fleeting or reduction of the dye so annoyingly characteristic of Janus green.

The films of stain were made in the usual manner by flooding clean, flamed slides with an absolute alcoholic solution of pinacyanol and draining off the excess. One drop of stock solution (0.1% dye in absolute alcohol) was used for every cubic centimeter of the same solvent. This dilution proved to be satisfactory for studying mitochondria in films of peripheral blood. Where many cells occur in smears such as in leukaemic blood or in spreads made from material obtained from spleen, bone marrow or lymph node punctures, it is necessary to increase the dye concentration. The strength of pinacyanol to procure an optimum staining of mitochondria must, for obvious reasons, be determined by each experimenter in a series of trial stains. Pinacyanol may be combined with neutral red for double stains and apparently does not inhibit the effect of the neutral red as does Janus green. The stain films should be made shortly

[1]Obtained from the Eastman Kodak Company, Rochester, New York.

[2]Proescher, F. 1933. Pinacyanol as a histological stain. Proc. Soc. Exper. Biol. and Med., **31**, 79–81.

before use since they do not seem to keep well altho the stock alcoholic solution, if kept well stoppered, does not deteriorate.

In a blood smear made upon a dye film of satisfactory concentration and sealed with salvoline, the mitochondria at first may be seen in some of the cells as untinted refractive rods or coccoid bodies which slowly assume a pale bluish color that increases steadily until it is intensely purple or blue. Mitochondria so stained have been observed unchanged in character two to four days later. In properly stained supra-vital smears, pinacyanol seems to be specific for mitochondria, since vacuoles, fat droplets and specific granules have not been observed to stain in the living cells.

It is true that the dye, like Janus green, is toxic and when too concentrated rapidly stains the nuclei and kills the cells. Pinacyanol tends to stain the nuclei of cells a lavender color even in optimum dilutions for demonstration of mitochondria. The nuclear tinting, however, is not necessarily a sign that death of the cell is imminent; since, as far as can be observed by comparison with unstained cells, the motility and behavior of the cell with a stained nucleus is unchanged for a surprising length of time. The polymorphonuclear neutrophiles appear to be affected first, especially those with the more highly segmented nuclei.

Likewise the nuclei of the other cells of the blood will become tinted after a time, but since the effect is so gradually produced, there is ample time for study of the cells. Even tho the nuclei may stain, the dye does not seem to alter their character as may be seen by comparing them with those of cells from unstained smears. In fact the nuclear staining is of considerable advantage since it offers a means of determining the relative fineness or coarseness of the chromatin masses in different nuclei. Such differences, when considered with other features of a given cell, aid materially in its identification. Since the dye is insoluble in protective saline solutions, such as those of Tyrode and Ringer, a satisfactory medium has not been found for perfusion of an animal with the dye for the staining of mitochondria in tissues before removal. Material from haemopoietic tissue, such as bone marrow which is ordinarily too thick to spread, may be thinned by a drop of serum or peritoneal fluid in which the dye is reasonably soluble.

# A COLLOIDAL SILVER METHOD FOR NERVE CELLS AND PROCESSES, NEUROGLIA AND MICROGLIA

RUTH STAGE, *Division of Anatomy, University of California Medical School, Berkeley, Calif.*

ABSTRACT.—**A method is described whereby nerve cells and processes, neuroglia and microglia may be stained using colloidal silver solutions (argyrol, silvol, 10% and 20%).**

**Fresh, unfixed brain tissue is stained in bulk in argyrol or silvol, and then dehydrated, embedded in low viscosity nitrocellulose, and sectioned. Before reduction the sections are treated with gold chloride to replace the silver. Sections are reduced in a formalin hydroquinone solution, fixed in sodium thiosulfate, dehydrated, and mounted in euparal. A method is described for removing the nitrocellulose before mounting.**

**No variation in the method was found to be necessary for the various species tested (rat, guinea pig, rabbit, and dog).**

The routine silver methods used for the study of the nervous tissues are for the most part modifications of the original Golgi and Cajal reduced silver and silver pyridine methods, and of the Bielschowsky ammoniacal silver methods. These methods and their modifications involve the impregnation of the nervous tissue with some inorganic silver salt or complex, the reduction and deposition of the silver in the nervous elements, and the subsequent replacement of the silver with gold.

In the method herein described two new principles are introduced, first, the use of colloidal silver for impregnation and second, the replacement of the silver with gold before reduction.

Taft and Ludlum (1930) have shown that fresh brain tissue can be stained by means of a silver proteinate (argyrol). Dieterle and Neumann (1930) claim that a colloidal effect can be obtained by reducing with dilute formalin sections stained with ammoniacal silver solutions. Recently Bodian (1936) has reported a method by which nerve processes and nerve endings can be stained with a silver albumose solution (protargol) to which metallic copper has been added. The one advantage of using colloidal silver for impregnation is that interfering precipitates are rare.

The silver is replaced with gold before reduction, first, because colloidal silver reduces slowly and with difficulty, second, because

## PLATE I

**Fig. 1.** Cerebral cortex of rabbit. 10% argyrol. × 85.

**Fig. 2.** Same as Fig. 1, showing large pyramidal cells. × 360.

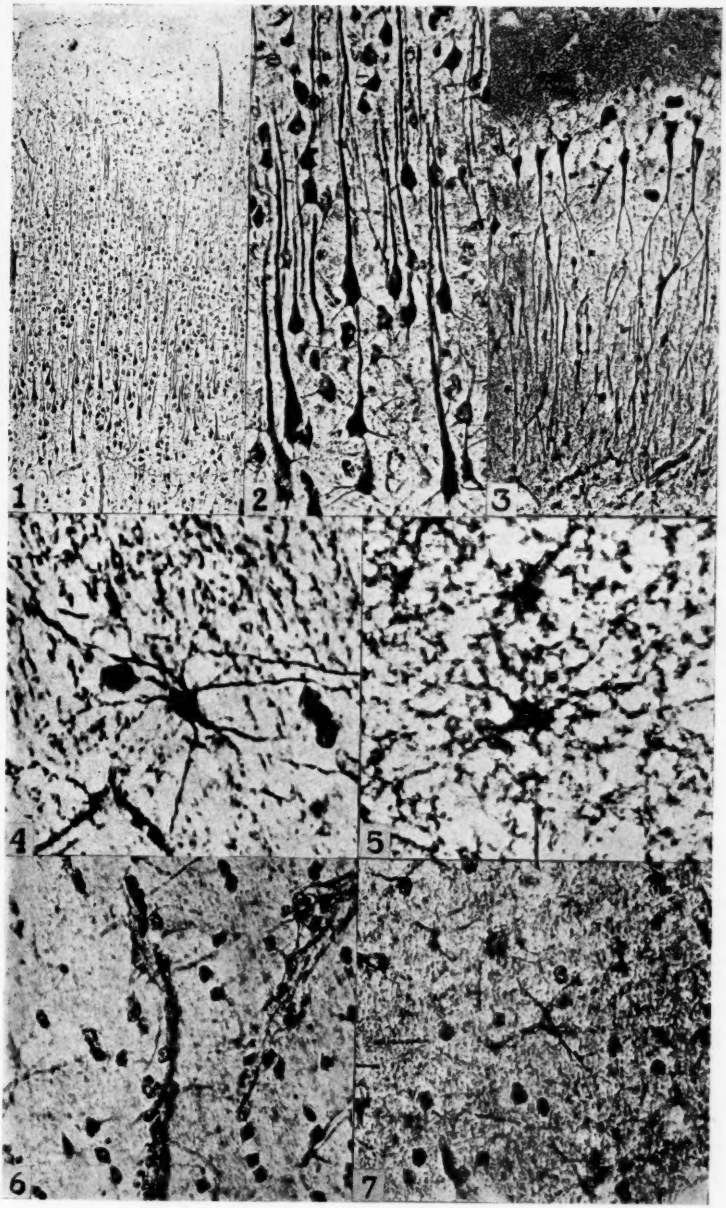
**Fig. 3.** Thalamus of rat, showing dividing processes of thalamic cells. 20% argyrol. × 200.

**Fig. 4.** White matter of cerebrum of rat, showing a fibrous astrocyte. 10% argyrol. × 1000.

**Fig. 5.** White matter of cerebrum of dog, showing a protoplasmic astrocyte. 20% silvol. × 1000.

**Fig. 6.** White matter of cerebrum of rat, showing perivascular satellites (oligodendroglia). 20% argyrol. × 440.

**Fig. 7.** White matter of cerebrum of guinea pig, showing microglia. 10% silvol. × 440.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such treatment affords a method of destaining tissue which has been overimpregnated, and in addition gives a lighter background, which makes for better contrast.

The method stains nerve cells and processes a dark rose color on a grayish white background; cortical and thalamic cells and their processes show up particularly well. It may also be used for the demonstration of neuroglia and microglia, which it stains dark rose or black; protoplasmic and fibrous astrocytes, perivascular satellites, and interfascicular oligodendroglia are all shown.

## Method

*1. Staining:*

Stain fresh brain tissue by placing thin slices (not over 3 mm. thick) in a freshly prepared colloidal silver solution (argyrol, silvol); it is necessary to have a large volume of solution: roughly 20 cc. for each sq. cm. of surface to be stained. The period of staining varies with the size of the pieces, the time since death, and the species of animal from which they were obtained; it varies from 48 hours to several weeks, and cannot be predicted. Overstaining, however, is of little consequence.

The tissue is stained when a freshly cut surface exhibits a dark brown color; and if a small piece is removed and crushed under a cover slip, nerve cells and processes will be found to be stained yellow.

*2. Dehydrating, embedding, sectioning:*

When the staining process is complete, remove the tissue and rinse quickly in 70% alcohol (2–3 min.), and then dehydrate, starting with 80% alcohol. (The colloidal silver diffuses out in the lower alcohols.) During dehydration allow three hours or over-night for each 5% step. From absolute alcohol place tissue in ether alcohol (ether and absolute alcohol, equal parts) for three to five hours, and then for one day each in 10%, 25%, and 50% low viscosity nitrocellulose.[1] Mount the pieces from 50% nitrocellulose on fiber blocks and harden in chloroform three to six hours. Leave in 85% alcohol (two changes) overnight and cut sections 10–20 $\mu$, receiving them in 85% alcohol. (The colloidal silver may diffuse out in the lower alcohols; the higher alcohols cause undesirable softening of the nitrocellulose.)

[1]R.S. ½ sec. low viscosity nitrocellulose, viscosity 3/20–4/20, manufactured by the Hercules Powder Company, Parlin, New Jersey.

In making up the solutions in this laboratory allowance is made for the alcohol content of the nitrocellulose:

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 10% | 10 g. | nitrocellulose, | 50 | cc. | ether, | 50 | cc. | absolute alcohol |
| 25% | 25 " | " | 55 | " | " | 45 | " | " " |
| 50% | 50 " | " | 60 | " | " | 40 | " | " |

*3. Treatment of sections with gold chloride, and reduction:*

Remove a section from 85% alcohol and float it on the surface of 0.5% aqueous gold chloride solution; the color of the section will change from yellowish brown to grayish white within one minute. As soon as this color change is complete, quickly remove the section and wash it rapidly in distilled water for 15 seconds, moving it back and forth by means of a glass hook. If, after final treatment a trial section is found to be too dark, wash succeeding sections longer than 15 seconds. For heavily stained sections the washing time may be 2 to 3 minutes, rarely longer.

After washing, place section immediately in a reducing solution of formalin and hydroquinone (hydroquinone, 0.3 g.; formaldehyde, neutral (Merck's) 30 cc.; distilled water, 70 cc.) for 2 to 4 minutes, rinse in distilled water 1 to 2 minutes, fix in 5% sodium thiosulfate 5 to 10 minutes, wash in distilled water 10 to 15 minutes, dehydrate in 95% alcohol 15 to 20 minutes, remove the nitrocellulose by means of ether alcohol, rinse off the ether alcohol with absolute alcohol, and mount in euparal.

To insure clarity and preservation of sections it is essential that the nitrocellulose be completely removed before the sections are mounted. This is most readily accomplished as follows:

a) Remove section from 95% alcohol by brushing it on to a piece of cigarette paper (non-ribbed variety) and smoothing out all wrinkles.

b) Apply the section to the slide, blotting it thru the cigarette paper with several thicknesses of filter paper.

c) By means of a brush apply absolute alcohol to the section thru the cigarette paper, brushing lightly back and forth. Blot with filter paper.

d) In the same manner apply several drops of ether alcohol.

e) Carefully peel off the cigarette paper (quickly, to avoid drying of section) and drop ether alcohol gently on the section until every trace of nitrocellulose is gone.

f) Rinse off the ether alcohol with absolute alcohol, wipe off the alcohol around the section, apply a drop of euparal and a cover slip.

By this method different tissue elements may be stained by varying the concentration of the colloidal silver solution. Thus, it has been found that:

(1) 10% argyrol stains nerve cells and processes and astrocytes.

(2) 20% argyrol stains nerve cells and processes, astrocytes, oligodendroglia and microglia.

(3) 10% silvol stains nerve cells and processes, astrocytes and microglia.

(4) 20% silvol stains nerve cells and processes, astrocytes and oligodendroglia.

*4. General precautions:*

a) The colloidal silver solutions should be freshly prepared before use. (Grind crystals to a powder and dissolve in distilled water.) Argyrol four or five months old stains very rapidly (probably because of the gradual liberation of free ionic silver), but is prone to produce disturbing precipitates.

b) Tissue should be kept in the dark during staining and dehydration.

c) Material may be kept in the colloidal silver several months without undue overstaining; but as soon as the tissue is removed from the silver solution the procedure as outlined above should be carried thru without interruption.

d) Sections should be manipulated only with glass hooks.

## REFERENCES

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DIETERLE, R. R. and NEUMANN, M. 1930. New demonstration of Hortega cells. Arch. Neurol. and Psychiat., **24**, 1154.

TAFT, A. E. and LUDLUM, S. D. 1930. A method for staining unfixed brain tissue with silver. Proc. Soc. Exp. Biol. and Med., **27**, 582.

## SIMPLE AIDS TO MICROSCOPE ILLUMINATION

D. B. O. SAVILE, *Abbotsford, Quebec, Canada.*

*A Mounting for the Reflecting Prism.* The somewhat delicate pivots of the microscope mirror mounting make it unsuitable for carrying the greater and unbalanced weight of the reflecting prism. For ease of adjustment the prism should pivot about the center of the reflecting plane, which is far from the center of gravity. Tight pivots and large bearing surfaces are needed to overcome the tendency to slip.

A suitable clamp of the type illustrated in the accompanying drawing can be constructed easily from fairly heavy gauge sheet copper or brass. First cut a strip to form the saddle A, which holds the prism; after bending the strip into a U just wide enough to slip over the prism, solder small distance pieces (shaded in the elevation view) inside the arms of the U to hold the face of the prism about ¼ in. away from the base of the saddle; this prevents the reflecting surface from being scratched and facilitates attachment of the pivots in the required place. The corners of the saddle are bent over at B to keep the prism in place. A small strip C is soldered on as shown to lend rigidity; it should preferably be made with a slight curve in the center; pressure on this curve spreads apart the arms of the U, facilitating removal of the prism for cleaning.

Bend another strip, for the clamp D, to the shape shown in the plan view. Make cuts about ⅛ in. deep into the lower side at the points E, E, turn up the metal as shown and run a little solder into the corners where the overlap occurs; this gives the necessary rigidity. The bearings, perhaps, present the chief difficulty. If a lathe is available, steel spigots G and brass or bronze collars H can quickly be turned up; otherwise one must make-shift with anything available. Personally, I used small brass terminal nuts from a dry cell and the tops of two broad-headed nails. A little work with a fine flat file resulted in a smooth fit. The finished spigots and collars are soldered in position. The soldering is best done without a soldering bit. Clean the surfaces to be joined, apply saturated zinc chloride, heat and coat with solder; finally apply more zinc chloride, superimpose the pieces and press together in the flame till the solder runs. In attaching the spigots to A the preliminary tinning should be done before the distance pieces are soldered on inside; when the actual joining is done, direct a pointed flame downward onto the spigot and

stop heating as soon as the solder flows; otherwise the distance pieces may become unsoldered.

The means of attachment to the microscope depends upon the make of instrument; a split plug of the type shown at J is commonly used. If the prism is always to be used with the same microscope,

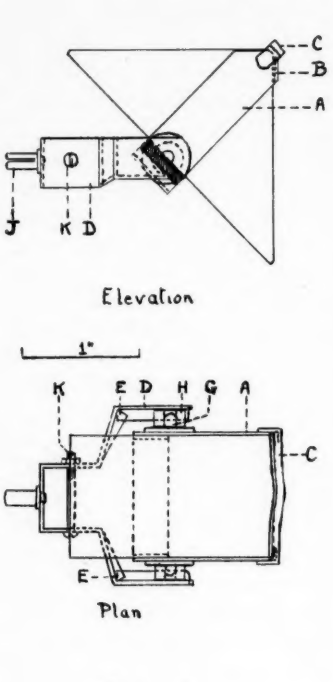



Fig. 1

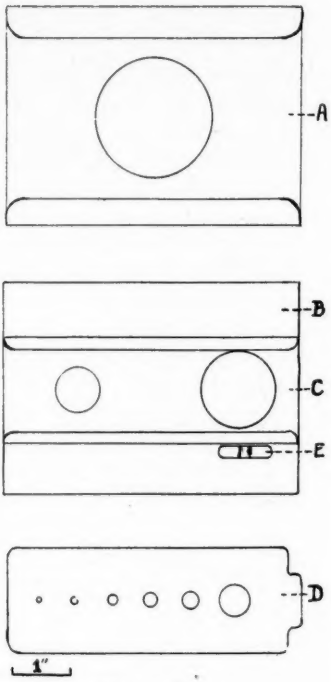



Fig. 2

Fig. 1. A mounting for the reflecting prism.

Fig. 2. Diaphragms for the microscope lamp.

the attachment of the mirror mounting may be used. Alternatively a split plug may be fitted and pressed into a hole in a hardwood block, the clamp D being swung down into a vertical position and the block being shaped to fit between the feet of the microscope stand; the only disadvantage of this method is that the prism does not move with the microscope. A suitable plug may be found on a derelict microscope or may be made by cutting down a pin from an electric

plug of the old type which had split pins as connectors in place of the flat strips commonly used today.

To complete the mounting, the clamp is drilled to take a small bolt K; the nut on this is tightened until the prism moves fairly stiffly. Finally it should be noted that alterations in some of the dimensions will be necessitated by different sizes of prism.

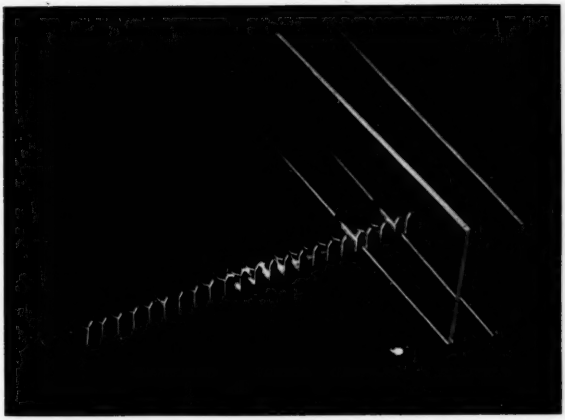
*Diaphragms for the Microscope Lamp.* It is convenient to have a reasonably compact set of lamp diaphragms which will allow a full range of stops without having to remove one slide and insert another or alter the relationship of lamp and condenser. The set here described has been found satisfactory and is simple to make. The necessary material was obtained from 9 × 12 cm. film pack cases, which are made from thin, easily-worked sheet metal. Part A (see Fig. 2) is made from the back part of the casing, the ends being trimmed and the sides being folded over to give a sliding fit over B, which is made from the piece which presses the films against the face of the pack. Part A is mounted on the lamp by whatever means prove most suitable for the type of lamp employed; it should, of course, be immediately in front of the ground glass. Holes are cut in B slightly larger than those in C; C is formed with the edges turned over to form guides for D and is then sweated onto B; its back and the corresponding front portion of B are rubbed bright with fine emery paper, the surfaces are painted with saturated zinc chloride, chips of solder are distributed over B, and C is placed on top. The two parts are placed in position on any convenient support, C is pressed firmly down and heat is applied from below with a bench burner until the solder flows freely; the pressure is maintained on C until the solder has set, when the whole is washed thoroly under the tap and dried. A small loop E is soldered on to B to form a handle. D is cut to slide in C and is turned up at the end for convenience of manipulation. The larger holes can be marked out by scribing circles with a pair of dividers and are easily cut out with a small pair of curved scissors. The smaller holes must be drilled: either sweat the piece D to a strip of scrap brass or clamp it between blocks of hardwood for the operation; the former is recommended as there is less likelihood of the holes being out of position. To avoid tearing such thin metal the sheet must be firmly held and the drill turned fast but fed very gently.

Before making any of the holes scribe center lines the full length of each part and make certain that these are superimposed in assembling; failure to get the various openings correctly aligned necessitates extra manipulation of the mirror.

The holes in D must be spaced so that when one is centered over the smaller of the two holes in C no light shows thru the adjacent ones. The sizes of openings required depend upon the objectives and condenser employed, but the range illustrated covers most requirements.

## NOTES ON TECHNIC

A Handy Slide and Cover Slip Rack.—The cartridges of 25 staples each, manufactured by the E. H. Hotchkiss Co. for their No. 1 Automatic Paper Fastener and available at most stationers', form a cheap and handy holder for clean slides and cover slips. The car



tridges may simply be laid on their backs on the table, or nailed to a small board. A shallow Stender dish, with a portion of a cartridge in the bottom, will serve as a Columbia staining jar for many purposes.—K. W. Cooper, Dept. of Zoology, Columbia University, New York, N. Y.

The Cellosolve-Nitrocellulose Technic.—The use of ethylene-glycol-monoethyl ether ("Cellosolve", Carbide and Carbon Chemical Co.) as a matrix solvent may be recommended in cases of difficult penetration.

For use with large blocks of nervous tissue the following procedure has been found satisfactory:

1. Fix and wash as usual.
2. Place block in several changes of cellosolve.
3. Run up the block in graded concentrations of "nitrocellulose ½-sec." (Hercules Powder Co.) to a fairly heavy concentration of solute. (The specimen may be left for an indefinite period at this stage without danger of drying up since the boiling point of cellosolve is quite high. This quality also inhibits bubble formation in ventricles and other "blind spaces".)

4. Transfer piece to alcohol-ether solution of "nitrocellulose ½-sec." in about 10% concentration. Thicken gradually, block up and harden in chloroform.

Any of the usual stains may be used after this method. It has been our custom to follow dehydration in 95% alcohol by removal to terpineol if the cellulose matrix is to be preserved. The sections are placed on the slide and flushed with xylene and then mounted in balsam. If the matrix is to be removed the section is placed from 95% alcohol on the slide and flushed with absolute alcohol and then methyl salicylate which removes the matrix and clears simultaneously. FRED A. METTLER, CECILIA C. METTLER AND F. C. STRONG, University of Georgia School of Medicine, Augusta, Ga.

THE DIOXAN PARAFFIN TECHNIC.—The cutting of sections of 2 by 3 inches square in paraffin can be accomplished with reasonable care by the substitution of 1, 4 diethylene oxide ("Dioxan", Carbide and Carbon Chemical Company) for alcohol and xylene in the embedding process.

For the embedding of large pieces of nervous tissue (and presumably other materials rendered friable by xylene) the following procedure is recommended.

1. Fix and wash as usual.
2. Place specimen in dioxan changing occasionally until anhydrous copper sulfate is no longer turned blue by the discarded dioxan. (The tissue may be conveniently left in this fluid for weeks.)
3. Embed in paraffin dissolved in dioxan in the same way in which one uses xylene or chloroform.
4. Section and mount.
5. Dissolve paraffin from section with dioxan and transfer to water. Stain as usual and mount.

This method possesses the advantage of preserving fine detail and keeping large blocks of the tissue soft.—FRED A. METTLER, F. C. STRONG AND CECILIA C. METTLER, University of Georgia School of Medicine, Augusta, Ga.

ALUM COCHINEAL-HEMATOXYLIN STAIN.—In using Delafield's hematoxylin for staining *toto* mounts the dark blue color of internal structures obscures many details; or if the destaining process is carried far enough to avoid this many structures completely lose their stain. Excellent slides have been made of trematodes and nematodes *in toto*, and of sectioned flatworms by using three parts of alum cochineal and one part of Delafield's hematoxylin. The recommended procedures for several different objects are:

| | Alum Coch. | Delaf. Hematox. | $H_2O$ | Time |
|---|---|---|---|---|
| Sectioned material | 3 pts. | 1 pt. | 10 pts. | 20–40 min. |
| Trematodes (*toto*) | 3 pts. | 1 pt. | 25 pts. | 2–3 hrs. |
| Nematodes (*toto*) | 3 pts. | 1 pt. | 50 pts. | 12–24 hrs. |

Tissues should be passed thru acid and alkaline alcohol as when using straight Delafield's. Since alum cochineal is not taken out as rapidly as the hematoxylin, one gets a beautiful contrast in colors —ranging from light pink to dark purple.

This stain should be used after fixatives containing acetic acid, such as Beauchamp's aceto-formo-alcohol. It usually deteriorates after a few days.—Bruce D. Reynolds, University, Virginia.

The Use of Acetates as a Means of Removing Air Bubbles from Lacto-Phenol Mounts of Fungi.—While working on species of *Chaetomium* difficulty was frequently encountered when the material was mounted in glycerin jelly or lacto-phenol because air bubbles would be held by the setae and the spores. This difficulty was overcome by adding a drop of methyl or ethyl acetate to the material. The spores and setae were wetted almost instantly.

It was found convenient to put a drop of Amann's lacto-phenol (lactic acid, 20 cc.; phenol, 20 g.; glycerol, 40 cc., and water, 20 cc.) on the slide; a bit of the fungus containing several perithecia was then placed on the slide in the lacto-phenol. The drop of methyl or ethyl acetate was then added, wetting all the perithecia. They could then be dissected in the lacto-phenol as the acetate evaporated rapidly and the lacto-phenol took its place, wetting the perithecia and spores. After dissection, a piece of glycerin jelly was placed on the slide, heated, and the mixture of glycerin jelly and lacto-phenol covered with a cover glass without the annoying presence of bubbles.

The low viscosity of the acetates combined with their low surface tension should be of value where great masses of spores are encountered which will not become wetted, when air bubbles are held in the mycelium, or when spores, by their presence on the surface of a liquid, obscure other features.—R. H. Tschudy, University of Wyoming, Laramie, Wyoming.

## STAINS RECENTLY CERTIFIED

**In the table below is given a list of the batches of stain approved since the last one listed in the July number of this Journal.**

STAINS CERTIFIED JUNE 1, 1936 TO AUG. 31, 1936*

| Name of dye | Certification No. of batch | Dye Content | Object of tests made by commission† | Date approved |
|---|---|---|---|---|
| Congo red | NQ 6 | 90% | As histological counterstain | June 30, 1936 |
| Wright's stain | LWr 7 | —— | As blood stain | July 14, 1936 |
| Methyl green | NG 11 | 80% | In histology and as constituent of Pappenheim stain | July 15, 1936 |
| Bismarck brown | NN 6 | 55% | As histological and bacteriological stain | July 18, 1936 |
| Crystal violet | NC 17 | 94% | As histological, cytological and bacteriological stain | July 22, 1936 |
| Crystal violet | LC 11 | 97% | As histological, cytological and bacteriological stain | July 22, 1936 |

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

# LABORATORY HINTS
## FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

*J. A. de Tomasi* — *Abstract Editor*

### BOOK REVIEWS

GUYER, MICHAEL F. **Animal Micrology.**, 4th ed. 6 x 9 in., 331 pp. Cloth. 76 illustrations. Index. University of Chicago Press, Chicago, Ill. 1936. $2.50.

This well-known book needs no introduction to workers in microscopic technic. The new edition is about 30 pages longer than the 3rd and has generally been brought up to date. Chapter VII of the new edition is entirely new, dealing with the dioxan technic, a procedure which has come into use in histology since the last edition appeared. Several minor changes with added material have been made thruout, especially in Chapter XVII on embryological methods, Chapter XVIII on cytological methods, and Appendix B which contains formulae of stains and various reagents.—*H. J. Conn.*

### MICROTECHNIC IN GENERAL

PETRUNKEVITCH, A. and PICKFORD, G. E. **On the relative acidity of histological fixing fluids.** *Anat. Record*, **65**, No. 4, 461–6. 1936.

The hydrogen-ion concentration of 34 standard fixing fluids was tested with a glass electrode and in several cases colorimetrically. With the exception of aq. osmic acid and 'neutralized' formalin none of the fluids have a pH higher than 4.9, while the most acid fluid, namely Gilson's sublimate mixture, gave a pH of about 0.0. The pH of mixtures containing formalin varies with that of the formalin used. Acidity as such, has nothing to do with fixation of mitochondria in view of the fact that Champy's fluid is quite acid (pH 1.5) and Flemming's without acetic still more so (pH 1.2).—*Author's Abstract.*

### DYES AND THEIR BIOLOGICAL USES

GIROND, A., LEBLOND, C. P., RATSIMANANGA, R. and RABINOWICZ, M. **L'acide ascorbique ou vitamine C dans la cellule et sa detection.** *Protoplasma*, **25**, (1), 115–23. 1936.

Szent-Gyōrgyi had shown that the suprarenal cortex macroscopically blackens with silver nitrate and attributed this reducing action to the presence of vitamin C. The authors have found that this reaction can be used histologically to detect vitamin C in the cells of various tissues. The method is not described in detail except that the silver nitrate is acidified and the specificity of the reaction is diminished by heat and light. The reaction results in the formation of blackened granules either about the Golgi bodies or distributed thru the cytoplasm. It occurs abundantly in the suprarenal cortex, corpus luteum, interstitial cells of the testes, and the glandular part of the hypophysis. When the guinea pig from which tissues are taken has been deprived of vitamin C in its diet, the histological picture gives indication of this deprivation. The silver nitrate reaction occurs only in the presence of active vitamin C.—*R. Chambers.*

GUILD, R. and RAPPORT, D. **Calorigenic action of methylene blue during muscular exercise.** *Proc. Exp. Biol. & Med.*, **34**, 459–61. 1936.

The intravenous injection of 10 mg. of methylene blue per kilo in a dog was followed by a 42 per cent increase in metabolism, predominantly at the expense of the oxidation of carbohydrate.—*M. S. Marshall.*

HANUT, C. and FAUTREZ, J. **Études sur les variations de dispersion des colorants.** *Protoplasma*, **23**, 93–108. 1935.

This study concerns the dispersibility of dyes in solution. Trypan blue in concentrations from 0.25 per cent to 2 per cent is relatively stable, an increase in concentration increases very slightly the dimensions of the particles but the solution does not tend to flocculate. The same is true of ammoniacal carmine, Ink R. A. L. (a specially prepared India ink), used by Girard and Cordier on nephric tubules. On the contrary, brilliant Congo R and particularly benzoazurine are completely peptized only in the more dilute solutions; above a critical point they tend to flocculate. Blood serum tends to increase the dispersion of the dyes mentioned in concentrations below 1 per cent; above that the particle size approaches that in aq. solutions. Other colloidal solutions, such as ovalbumin, diluted 100 times, dextrinea 0.1% and gum arabic 0.05 per cent definitely decrease the dispersion.—*R. Chambers.*

LISON, L. **Une réaction micro et histochimique des esters sulfuriques complexes, la "réaction metachromatique".** *Bull. Soc. Chim. Biol.*, **18**, 225–30. 1936.

Sulfuric acid esters of high molecular weight (e.g., chondroitin sulfuric acid of cartilage) change the color of very dilute solutions (1:10000 to 1:50000, solvent not given) of certain metachromatic dyes, among which are toluidine blue and brilliant cresyl blue. This "metachromatic color change" is an extremely sensitive and strictly specific test for these substances and their salts. It must be distinguished from the "ionic color change" caused by alkaline solutions. The author gives 2 ways of distinguishing these color changes. (1) Extraction by a hydrocarbon: shake the solution which has changed color with a little benzene or toluene; if the solution becomes colorless while the solvent becomes colored, it indicates an ionic color change, whereas if the solution remains colored, it is a case of metachromatic color change. (2) Action of heat: raising the temperature changes the equilibrium between the 2 forms of the dye in favor of the "normal" form at the expense of the metachromatic one and does not affect the equilibrium between the ionic forms. The former is reversible and the color reverts to the metachromatic form on cooling. The sensitivity of the "metachromatic reaction" is thus increased for weakly chromotropic substances by cooling the test solution before use. The sensitivity of the reaction diminishes with decreasing molecular weights of the esters.—*L. Farber.*

MILLS, M. A. and DRAGSTEDT, C. A. **Bromsulphalein dye retention rest as a measure of functional activity of reticulo-endothelial system.** *Proc. Soc. Exp. Biol. & Med.*, **34**, 228–31. 1936.

Functional impairment obtained by blockading the reticulo-endothelial system was measured by injecting dogs with 2 mg. of bromsulphthalein per kilo of body weight intravenously and taking blood samples 5 and 30 min. later. The dye was detected in plasma by adding 2 drops of N/10 NaOH. If the reaction was obscured by reagents used for blocking precipitation, acetone was used before alkalinizing. Dye retention appeared to give a direct measurement of the effectiveness or degree of blockading.—*M. S. Marshall.*

## ANIMAL MICROTECHNIC

BROWN, A. F. and KRAJIAN, A. A. **Histologic demonstration of uremia by precipitation of xanthydrol urea in tissues.** *Arch. Path.*, **21**, 96–9. 1936.

An excess of urea in the tissues may be demonstrated by the production of xanthydrol urea crystals by the following technic: Cut thin (2–3 mm.) blocks of tissue. Immerse 5–6 hr. in fresh solution of xanthydrol (5 g. in 100 cc. glacial acetic acid). Wash in running water 1 hr. Fix in 10% formaldehyde 6 hr. (Rapid fixation by heat may be used.) Wash in tap water. Cut frozen sections

5–10 μ. Transfer to glass slide. Pour several drops of dehydrated alcohol over the section from dropping bottle and blot. Repeat process. Cover with thin pyroxylin (celloidin). Fix by blowing over slide. Stain in a 1% aq. solution of eosin for several mimutes. Wash in tap water. Dehydrate 3 times with abs. alcohol. Place in carbol xylene for 2 min. Clear in 2 changes of pure xylene, 1 min. each. Mount in dammar.—*Lall G. Montgomery.*

CHANG, M. **A formol-thionin method for the fixation and staining of nerve cells and fiber tracts.** *Anat. Record,* **65,** No. 4, 437–42. 1936.

The nervous tissue can be fixed and stained simultaneously in 0.1 or 0.5 g. of thionin (Grübler) dissolved in 100 cc. of 10% formaldehyde for a few days up to 2–3 months. Then it is rinsed with dist. water, dehydrated in graded alcohol and embedded either in paraffin or in celloidin according to the usual technic. In so doing, the fiber tracts are stained red and cell bodies blue. Such a differentiation turns out more brilliantly if both fixation and dehydration be much prolonged.—*Author's Abstract.*

LISON, L. **Sur la recherche histochimique des oxydases par la réaction du bleu d'indophénol. Le cas des lipides.** *Bull. Soc. Chim. Biol.,* **18,** 185–9. 1936.

The blue coloration taken on by certain fatty substances on treatment with the Nadi reagent (α-naphthol + dimethylparaphenylene-diamine) is not due to a selective action by the indophenol blue in the reagent resulting from its oxidation by atmospheric $O_2$. It results from an oxidation of the Nadi reagent by the fatty substances themselves, or to be more exact, by a substance contained in the fatty material. In this case, however, it is not a question of an oxidase but of an oxidizing substance, very probably an organic peroxide, formed by the oxidation of unsaturated lipids. This fact calls attention to the possibility that the Nadi reaction may not always specifically indicate the presence of an enzyme.—*L. Farber.*

NARAT, J. K. **A new method for preparing multicolored corrosion specimens of the kidney.** Trans. of Chicago Path. Soc., Oct., 1935. *Arch. Path.,* **21,** 120. 1936.

After the blood vessels and ureter of the kidney have been thoroly flushed with water, they are filled with stained solutions of a vinyl resin in acetone. The organ is immersed in water for 24 hrs., a procedure which solidifies the injected solutions. The kidney is placed in conc. commercial hydrochloric acid for 4 days. All the tissues are digested and only casts of the blood vessels, ureter and calices containing the injected substances remain. By employing acid-fast dyes, multicolored specimens may be obtained.—*Lall G. Montgomery.*

SCHOUR, I. **Measurements of bone growth by alizarin injections.** *Proc. Soc. Exp. Biol. & Med.,* **34,** 140–1. 1936.

The rate of apposition of bone can be followed by the use of injections of sodium sulphalizarate. Rats 3 months old were given 0.5 cc. of 2% alizarin red S, C. I. No. 1034, (Coleman and Bell Co.) 4–6 days apart and were killed 2 days after the last injection. The dose ranged from 39–62 mg. per kg. Microscopic examination of ground transverse sections of the mid-root level of upper molar teeth showed a red line in the alveolar bone that was calcifying at the time of each injection.—*M. S. Marshall.*

## MICROÖRGANISMS

BROOKE, J. W. **Possible bipolar nuclear distribution in bacteria.** *Proc. Soc. Exp. Biol. & Med.,* **34,** 185–8. 1936.

Definite granules were demonstrated in a number of bacteria grown in the presence of sugars, altho some of the sugars were not utilized by the organisms tested. With a wax pencil, mark a circle on a slide prepared as for a flagella stain and add 2 loopfuls of sterile water followed by a loopful of culture. Air dry, and flood with a 10% alc. basic fuchsin. Keep moist with drops of 95% alcohol for 7–10 min. Wash with water, place a drop of acid alcohol at the periphery of the ring and immediately wash it off with a gentle stream of water. Air dry and

examine. With *Proteus vulgaris*, methylene blue, crystal violet, carbol fuchsin, 0.1% basic fuchsin and malachite green failed to give granule stains equal to those secured with 10% basic fuchsin.—*M. S. Marshall.*

BUTT, E. M., BONYNGE, C. W. and JOYCE, R. L. **The demonstration of capsules about hemolytic streptococci with India ink or azo blue.** *J. Inf. Dis.*, **58**, 5–9. 1936.

While India ink has been used often in the past for outlining bacterial capsules and organisms, no consideration has been given to the action of diluents upon the size of the capsular zone. An improved India ink technic is given with azo blue as an alternate choice. An imported product, Peliken ink No. 541 (Gunther Wagner) is used; Higgins waterproof ink can be used only after centrifuging. The hemolytic streptococci are grown on plates of veal infusion agar (pH 7.2) with 5% defibrinated sheep blood and 20% ascitic fluid added. Incubate 7–8 hr. at 35° C. Make a thin suspension of the organism in 1 drop of 6% dextrose on a clean slide, add 1 small drop of the ink, mix, and spread by using the edge of another slide (as in making a blood film). Dry and counterstain with 1:1 stock methylene blue solution (no further details specified) and methyl alcohol, or fix in methyl alcohol and counterstain with an aq. solution of any dye selected. Wash off the excess stain and allow to dry without blotting. Azo blue (Coleman and Bell) is used in the same manner in the form of a 1% solution in 6% dextrose. It gives larger capsular zone, but a coarser background.—*J. A. de Tomasi.*

CLAUBERG, K. W. **Ueber einige ergänzende technische Einzelheiten der neuen Indikator-Tellurplatte zur makroskopischen Diphtherie-bazillen-diagnose.** *Zentbl. Bakt., I Abt. Orig.*, **135**, 529–30. 1936.

Some difficulty has been experienced recently in connection with the new "indicator-tellur" plating medium (Clauberg, Zentbl. Bakt., I Abt. Orig., **134**, 271. 1935) for the recognition of diphtheria organisms macroscopically. The trouble is with the indicator (water blue). Instead of a uniform marine (navy?) blue when the medium is acid, the color often turns dark reddish violet. After incubation, the plate becomes dark dirty green which makes differentiation of the diphtheria organism difficult. In coöperation with the firm Dr. G. Grübler & Co., Leipzig, a new C. P. preparation has recently been developed called "Standardized water blue 6B extra P" which is stronger than previous products so that it can be used in a stock concentration of 1.75% instead of 2%. A deep blue circle or halo is characteristic of a diphtheria colony.—*J. A. de Tomasi.*

FUKUMOTO, K. **Studies on the vital staining of bacteria. I. Influence of pigment on the growth of tubercle bacilli and non-pathogenic acid-fast bacilli cultivated in Kirchner's medium to which Ajinomoto was added.** *J. Orient. Med.*, **23**, 34. 1935.

This is a report on 12 dyes investigated as to their bacteriostatic effects upon the tubercle organism and other acid-fast bacteria. Safranin and trypaflavin proved most toxic, while methylene blue, gentian violet, eosin, fuchsin and scarlet red were less so in the order named. (*Cited from* Zentbl. Bakt., I Abt., Ref., **121**, 310. 1936.)—*J. A. de Tomasi.*

FUKUMOTO, K. **Studies on the vital staining of bacteria. II. Pigment affinity of tubercle bacilli and non-pathogenic acid-fast bacillli.** *J. Orient. Med.*, **23**, 35. 1935.

Using for comparison tubercle bacilli and non-pathogenic acid-fast organisms, 20 dyes were tested for their bacteriostatic properties and stainability of the granules. It was found that most of the strains including the tubercle bacillus, could be stained vitally since growth continued long after adsorption of the dye had become evident. (*Cited from* Zentbl. Bakt., I Abt., Ref., **121**, 311. 1936.)—*J. A. de Tomasi.*

GUITTONNEAU, G. and BRIGANDO, JEANNE. **Sur une résistance aux colorations de certains corps microbiens acquise par chauffage dans le lait.** *Compt. Rend. Acad. Sci.*, **202**, No. 19, 1622–4. 1936.

Most of the bacteria in a sample of milk which has been submitted to considerable heat (1 hr. at 100° C. or 20 min. at 120° C.) will not stain but appear as white spots against the colored background. This can be shown with 1% aq. methylene blue, as well as with gentian violet, Unna's blue, erythrosin, or hematoxylin. It is thought that this particular behavior is dependent upon certain unknown conditions of the cells, and upon the development of definite sensitizing factors brought about by thermic alteration of the phosphocasein complexes in raw milk. —*J. A. de Tomasi.*

JOHNSON, C. M. **A rapid technique for iron-hematoxylin staining requiring no microscopical control of decolorization.** *Amer. J. Trop. Med.*, **15**, 551–3. 1935.

This new technic, suggested as a rapid stain for amoebae, includes the following steps: Fix 10 min. in sat. Schaudin solution, plus 5–10% acetic acid; treat 5 min. in iodized alcohol (95% alcohol plus $I_2$ until of a port wine color); wash in 70% alcohol, 5 min.; rinse in water, 1–3 min.; soak in 4% iron alum, 15 min.; rinse in water, 1–2 min.; stain with 0.5% aq. iron hematoxylin, 10 min.; decolorize with 0.25% iron alum; wash in running water and dry.—*J. A. de Tomasi.*

KEH-HUNG, LI. **Antigenicity of staphylococcal toxin detoxified by the photodynamic action of methylene blue.** *Proc. Soc. Exp. Biol. and Med.*, **34**, 659–61. 1936.

Grübler's methylene blue was used to detoxify staphylococcus toxin prepared by a modification of methods reported by Parker and by Dolman. Minute quantities of stock dye solution were added to filtrates and these were exposed on ice 20 cm. below a 100-watt lamp. Treatment removed the hemolytic, necrotizing and killing properties of the toxin but did not destroy the antigenicity of the product.—*M. S. Marshall.*

ONO, K. **Studien über die Färbung der Spirochaete pallidae, insbesondere über den Zusammenhang der Färbung mit den Vorbehandlungen. I. Einfluss der Vorbehandlung mit verschiedenen Fixierungs- und Beizmitteln auf die Färbbarkeit der Spirochaete pallidae und der Blutelemente.** *Lues, Bull. Soc. Japon. Syphiligr.*, **11**, 71–97. 1934.

The most satisfactory technic for staining the spirochaetes of syphilis in frozen sections is stated to be the following: Fix the tissue 90 min. to 24 hr. in 10% formalin, wash in tap water and cut. Transfer sections to a slide, dry at room temperature, fix again for 15 min. in the same strength formalin, wash thoroly, treat 15 min. in 1% $NaHCO_3$, and wash thoroly again. Stain 5–15 min. with 0.2–0.5% solutions of any one of the following dyes: Crystal violet, dahlia, extra violet, gentian violet, methyl violet, marine blue, or Victoria blue. (*Cited from* Zentbl. Bakt., I Ref., **120**, 345. 1936.)—*J. A. de Tomasi.*

ONO, K. **Studien über die Färbung der Spirochaetae pallidae, insbesondere über den Zusammenhang der Färbung mit den Vorbehandlungen. III. Die Färbemethoden der Spirochaete pallidae.** *Lues, Bull. Soc. Japon. Syphiligr.*, **11**, 247–62. 1934.

Smear preparations of *Spirochaeta pallida* were fixed 15 min. with 10% formalin or any of the 3 preferred fixatives mentioned in paper No. 1 of this series, washed thoroly in tap water and fixed another 15 min. in either 3% $H_2O_2$ or 1% $KMnO_4$. Various stains were tested by staining for 2–10 min. with 0.2–1%, aq. solutions. Particularly good staining was obtained when the formalin fixation was followed by washing, soaking for 5–15 min. in 1% $NaHCO_3$ and washing again. The following dyes gave the best results: 1% ethyl violet (5 min.), 1% crystal violet (15 min.), 1% dahlia (15 min.), 0.2–1% gentian violet (2–15 min.), 0.2–1% sky blue (2–15 min.), 0.2–1% marine blue (2–15 min.), 0.2–1% methyl violet (2–15 min.), and 0.2–1% Victoria blue (2–15 min.). (*Cited from* Zentbl. Bakt., I Ref., **120**, 345–6. 1936.)—*J. A. de Tomasi*

ONO, K. **Färbung der Spirochaetae pallidae in Gefrierschnitten.** *Lues, Bull. Soc. Japon. Syphiligr.*, **12**, 82–5. 1935.

This is the first of a new series of studies on the *Spirochaeta pallida*. The first series, dealing with the recurrent spirochaetes, was reviewed in this journal. The author studied the fixing and mordanting properties of 20 solutions and the staining properties of 31 dyes. It is shown that the staining is largely dependent upon the pre-treatment of the slide. Of all fixing and mordanting solutions, 10% formalin, 3% $H_2O_2$, and 1% $KMnO_4$ are preferred because the staining intensity of blood serum, proteins and erythrocytes is greatly reduced, assuring a strongly selective stain for the spirochaetes. Good results are also obtained with: 1:1 alcohol-ether, 5% alum, 5% $(NH_4)_2MoO_4$, 5% $K_2Cr_2O_7$, Müller's and Orth's mixtures. (*Cited from* Zentbl. Bakt., I Ref., **120**, 346. 1936.)—*J. A. de Tomasi.*

SCHMIDT, W. **Versuche zur Färbung von Virusarten mit Viktoriablau.** *Zentbl. Bakt., I Abt. Orig.*, **136**, 260–3. 1936.

Victoria blue 4R, a dye of the triphenylmethane series, has been used successfully by Herzberg (Zentbl. Bakt., I Abt. Orig., **130**, 183–326, 1933–34; **131**, 358, 1934) for staining virus preparations. In the case of smallpox, contact smears from infected cornea, testis, spleen, liver, kidney, and skin of rabbits and guinea pigs show elementary bodies distinctly present in great number. With the herpes virus, preparations from the cornea reveal light blue to violet bodies or particles, smaller than these elementary bodies but not so well defined. Negative results are recorded with vesicular stomatitis, foot and mouth, and fowl plague viruses. It is argued that these failures might be due to the much smaller size of the virus particles.—*J. A. de Tomasi.*

VERONA, O. **Manière de se comporter des microrganismes vis-à-vis de certaines substances colorantes. Étude particulière sur le vert malachite sur son application éventuelle en phytothérapie.** *Boll. Sez. Ital. Int. Micro.*, **7**, 426–8. 1935.

A list of 26 common dyes including a few acid-base indicators were tested as to the bacteriostatic effects upon a large collection of microörganisms including bacteria, yeasts and molds, in dilutions ranging from 1:1,000 to 1:1,000,000. Even at the highest concentration the action of most of them proved to be very weak. The only exceptions were malachite green and, in a lesser degree, brilliant green and gentian violet. Germination of seeds was neither retarded nor stimulated by a 1:10,000 malachite green medium; but it seemed to be an obstacle to the further development of plants. It also proved to be a better fungicide than $CuSO_4$ or lime on wheat seed. Alkalies, such as KOH, convert it into the leucobase which appears to be a stronger disinfectant than the original dye.—*J. A. de Tomasi.*

WALKER, A. W. **Comparison of precipitating action of basic dyes on bacteriophage and bacterial proteolytic enzymes.** *Proc. Soc. Exp. Biol. and Med.*, **34**, 726–8. 1936.

Precipitation of the bacteriophage and proteolytic enzyme was accomplished by adding, to 1 cc. of anti-coli phage in synthetic medium and to 1 cc. of the filtrate from a 4-day culture of *Pseudomonas pyocyanea* in synthetic broth, 7 cc. of saline and 2 cc. of M/70 dilutions of various dyes. The mixtures were allowed to stand 18 hr. at room temp. No appreciable inhibiting effect was noted with any dye, except pyronin. The active agents were present in precipitates but not filtrates prepared with safranin, neutral red, Janus green, thionin, methylene green and toluidine blue. They were present in both precipitates and filtrates prepared with brilliant cresyl blue, cresyl violet, gallocyanin, pyronin and malachite green.—*M. S. Marshall.*

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